

Effect of Asbestos on the P388D1 Macrophagelike Cell Line: Preliminary Ultrastructural Observations

by Neil F. Johnson* and Reginald Davies*

The ultrastructural effects of mineral dusts on a macrophagelike cell line (P388D1) were studied by transmission electron microscopy. The cells were found to contain virus particles which probably did not exert a cytopathic effect toward the cells. During mitosis the asbestos fibers appeared to be pushed to the periphery of the cell. The distribution of the asbestos in the P388D1 cells was similar to that seen previously in cultured peritoneal macrophages, and it was concluded that the cytotoxic effect of asbestos towards the cells was due to mechanical effects, e.g., by penetrating structures such as the nucleus or phagosomes, by preventing the ordered movement of organelles with the cell or by disrupting the cytoskeletal framework of the cell.

Introduction

Asbestos can induce the release of both lysosomal and cytoplasmic enzymes from cultured murine peritoneal macrophages (1) and the P388D1 macrophagelike cell line (2). A recent investigation (3) has shown that the release of lysosomal enzymes may result from the partial ingestion of the asbestos fibers and the incomplete fusions of the phagosome membrane, so allowing the release of enzymes into the medium from lysosomes which have fused with the phagosome. The cytotoxic activity of asbestos exhibited by the release of lysosomal enzymes may result from the fibers penetrating intracellular structures such as the nucleus and lysosomes, by preventing the movement of organelles within the cytoplasm or by disrupting the cytoplasmic organization provided by microfilaments and microtubules (3). The P388D1 macrophage cell line may provide a quicker and more reproducible test system (4) than the use of primary macrophage cultures. This communication examines the ultrastructural effects of asbestos on the permanent macrophage cell line (P388D1) and compares it with primary macrophage cultures to see whether the ultrastructural pathology of the cells is similar.

Culture of P388D1 Cells with Dusts

The P388D1 cell line was obtained from Dr. I. P. Gormley, IOM, Edinburgh, and was originally isolated by Dawe and Potter (5) from a methylcholanthrene-induced lymphoid neoplasm (P388) of a DBA mouse.

Stock cultures were maintained in plastic tissue culture Nunc flasks (174 cm² area, Gibco Ltd., Glasgow). The maintenance medium (100 cm³) used was Dulbecco's modified Eagle medium (Gibco Ltd.) containing 3.7 g NaHCO₃/L, 60 mg sodium benylpenicillin/L, 100 mg streptomycin sulfate/L and 10% newborn calf serum (Gibco Ltd.) which had previously been heat-inactivated (56°C for 30 min). The cultures were maintained at 37°C in an 8% CO₂/92% air atmosphere.

P388D1 cells were obtained from the stock cultures by vigorous shaking of flasks and the cells sedimented by centrifugation at 1000 rpm for 10 min. The cells were resuspended in phosphate-buffered saline (PBS) and an aliquot was added to 0.2% trypan blue in PBS and counted to determine the number of viable cells obtained. The cells were sedimented by centrifugation and resuspended in the culture medium. This medium was similar to the maintenance medium; however, the serum used had, in addition to being heat inactivated, been acid treated (6).

Cells (0.4×10^6) in 2 cm³ culture medium were added to each well (area of 4.5 cm²) of Limbro tissue

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culture multiwell plates (Flow Ltd., Irvine, Scotland) and left at 37°C in an 8% CO₂/92% air environment for 24 hr. For electron microscopy, 50,000 or 100,000 cells in 0.25 cm³ culture medium were added to the tops of Beem (USA) polyethylene embedding capsules (the tops being used as mini wells) and maintained as above.

Appropriate quantities of the dusts were autoclaved as dry powders in glass bottles and added to fresh amounts of culture medium at a dust concentration of 60 µg/cm³. The original culture medium was removed, and 2 cm³ "dusted" medium added to three wells (for each dust) of the multiwell plates and 0.25 mL added to the "Beem" tops; where these tops had received 100,000 cells, twice the dust concentration was used. Both the multiwell plates and tops were maintained for 18 hr at 37°C in an 8% CO₂/92% air atmosphere.

The culture medium was removed from the multiwell plates, and 2 cm³ "cell lysing" solution (Eagles balanced salt solution (Gibco) containing 1.65 g NaHCO₃/L, 1.0 g bovine serum albumin and 1.0 g Triton X100) was added to the wells. These were ultrasonicated to disrupt the remaining adherent cells, the cell lysates and culture medium were centrifuged at 2000 rpm for 10 min and the supernatants assayed for lactate dehydrogenase (LDH) by using the continuous flow fluorimetric method of Morgan et al. (7). The % LDH released into the culture medium was calculated as follows:

$$\% \text{ LDH in medium} = [M / (M + C)] \times 100$$

where *M* = LDH activity in the culture medium and *C* = LDH activity in cell lysates.

The mineral dusts used were DQ12 quartz (8) (obtained from IOM, Edinburgh), UICC Canadian chrysotile and crocidolite (9) and magnetite (BHD Chemicals, Dorset).

Electron Microscopy

At the end of the culture period the Beem tops were washed with PBS and fixed according to the method of Johnson and Davies (3) and examined by electron microscopy.

In addition, pellets of cells were taken and conventionally processed for electron microscopy with the exception that the post fixation step with osmium tetroxide was omitted. Sections were placed either on gold or copper grids and stained according to the method of Bernhard (10) to demonstrate RNA or Peters and Giese (11) to demonstrate DNA. Additional tops from nondusted and chrysotile-dusted cultures were fixed in sodium cacodylate-buffered glutaraldehyde prior to incubation to demonstrate

acid phosphatase activity (12) and then conventionally processed for electron microscopy.

Results

Untreated Cultures

The cells generally formed a monolayer but in some areas the cells were two or three deep. The cells were semicircular or semielliptical in cross section (Fig. 1a) with a surface possessing few microvilli and associated with only an occasional coated vesicle. Cell junctions could not be identified between adjacent cells. In some cell cross sections, c-type particles could be seen forming at the cell surface (Fig. 1d) and the cytoplasm contained virus particles (Figs. 1c, and 1e). The core of a proportion of the viruses stained positively for RNA (Fig. 1c) but not for DNA. The cytoplasm contained small round or tubular mitochondria, lipid-containing vesicles, cisternae of rough endoplasmic reticulum and a large round nucleus with diffuse chromatin and often a prominent nucleolus. In many of the cells, lysosomes were not a prominent feature; however, when they could be identified they showed a considerable variation in size. In the cultures stained for acid phosphatase activity, positive granules were identified (Fig. 1f). A small number of the cells were in mitotic division an infrequent finding was the presence of lysed cells.

Magnetite-Treated Cultures

The treated cells appeared ultrastructurally similar to the nondusted cells apart from magnetite-containing vesicles (Fig. 2a). Not all the cell cross sections contained dust particles.

Quartz-Treated Cultures

Quartz particles were found in the majority of cell cross sections. The particles were found within the cells (Fig. 2b) in membrane-bound vesicles (Fig. 3c) and apparently free in the cytoplasm (Fig. 3a). Quartz particles were also seen adjacent to the cell surface (Fig. 3b). The organization of the majority of cells showed little difference ultrastructurally to the control cells. However, there were a greater number of lysed cells in comparison to the nondusted cultures. There were also a small proportion of aberrant cells which possessed an electron-translucent cytoplasm containing distended cisternae of rough endoplasmic reticulum and mitochondria. The quartz particles were frequently associated with cutting artifacts, and it was difficult to distinguish the exact location of particles within the tissue, especially in relation to the membranes of enclosing vesicles.

Chrysotile-Treated Cultures

The majority of the cells appeared to be unaffected ultrastructurally by the presence of chrysotile fibers (Figs. 4a and 4b). There were more lysed cells compared to the control cultures, but fewer than in the quartz-treated cultures. Chrysotile fi-

bers were found partially engulfed by the cells, and the adjacent membranes were often associated with the presence of coated vesicles (Fig. 5b). Fibers were also found within phagosomes and penetrating from phagosomes. The cultures stained for acid phosphatase activity showed many positively

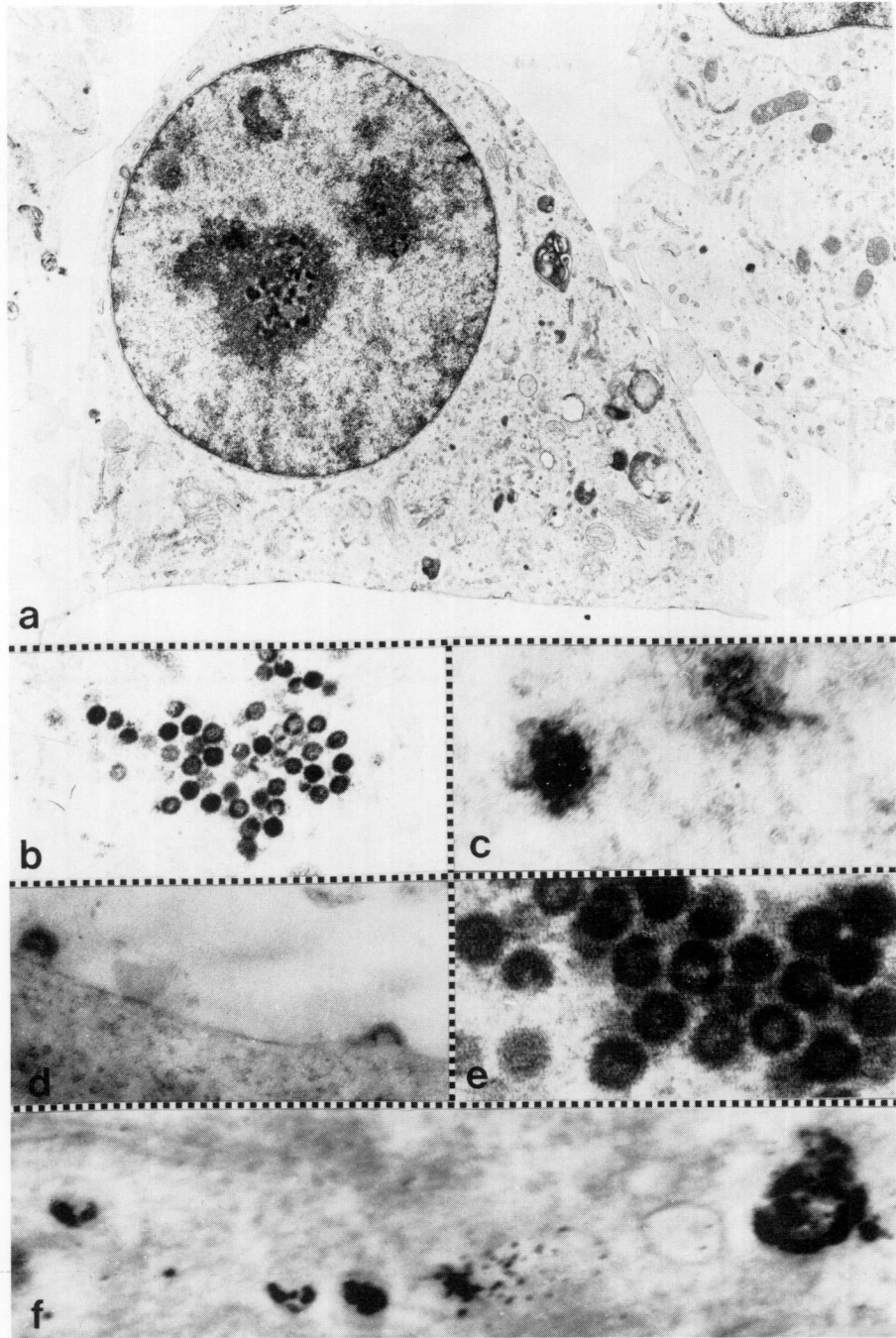


FIGURE 1. Electron micrographs of nondusted cells showing (a) the general cell organization ($\times 4,000$) and (b, e) the cytoplasmic virus particles (b, $\times 20,000$ and e, $\times 70,000$), some of which (c, stain positively for RNA ($\times 18,000$); (d) the cell membrane is associated with C type particles ($\times 40,000$) and (f) the cytoplasm also contains acid phosphatase-positive granules ($\times 35,000$).

stained vesicles containing fibers (Fig. 5c). Fibers were also present free in the cytoplasm (Fig. 4b), penetrating the nucleus or contained within the nucleus (Fig. 5a). On a number of occasions fibers were seen in invaginations which went deep into the nucleus (Fig. 6c) and, in cross sections, these fibers could be seen to be separated from the chromatin

by the nuclear envelope (Fig. 6b). Nuclear bodies were more frequently encountered in these cells than in the control cultures. Nuclear abnormalities were also more evident; these consisted of small out-pushings of chromatin material from the nuclear pore region and out-pushings from the nucleus involving both membranes of the nuclear envelope.

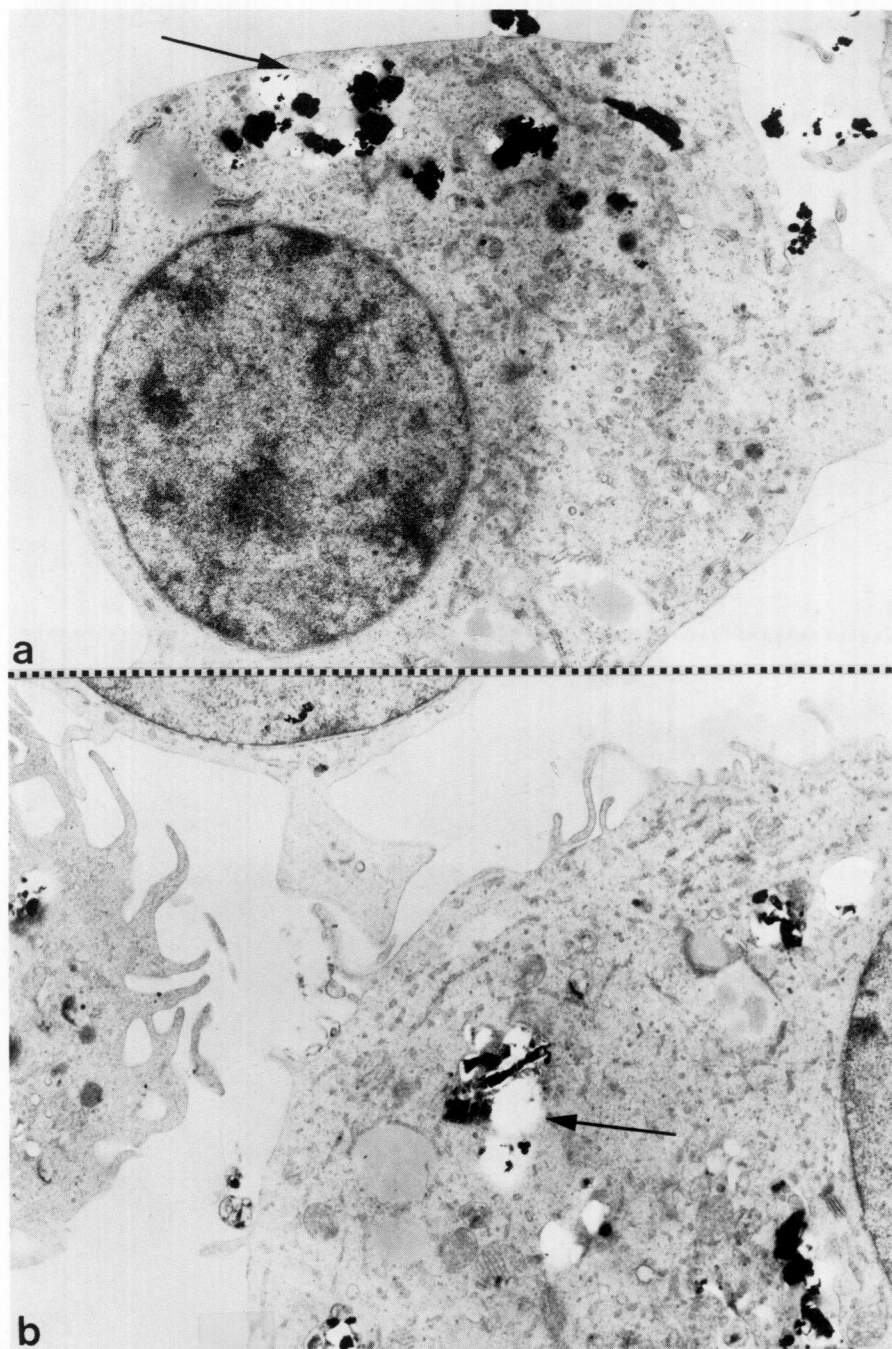


FIGURE 2. Electron micrographs of (a) a magnetite-treated cell ($\times 4,000$) and (b) a quartz-treated cell ($\times 16,000$). In both cases the normal architecture is preserved. Cutting artifacts are evident with both particles (arrows).

The shape of many nuclei was distorted and irregularly shaped (Fig. 6a). The nuclear indentations were often associated with adjacent chrysotile fibers. An unusual feature of the cytoplasm was the presence of large electron-dense irregularly shaped granules containing or partially containing fibers. In the few cross sections of mitotic figures encountered, the fibers were found in the periphery of the cells and

not intermingled among the condensed chromosomes. A small number of the cells containing fibers were abnormal and contained small mitochondria with an electron-dense matrix; the cytoplasm was electron-translucent and contained distended cisternae of rough endoplasmic reticulum. The nuclear envelope was frequently distended and the chromatin diffuse.

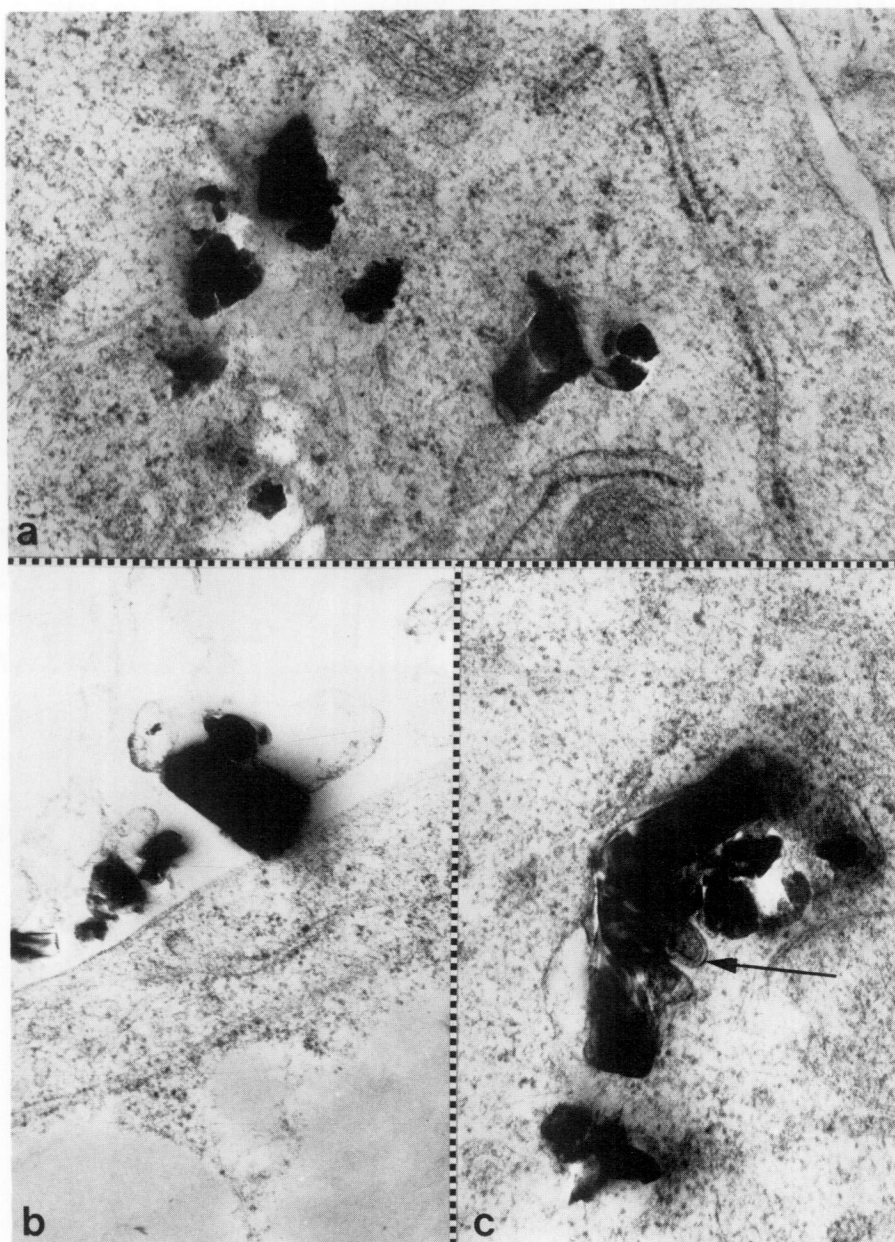


FIGURE 3. Electron micrographs of intracellular and extracellular quartz particles: (a) the apparently free cytoplasmic particles produce little if any cellular reaction, and the mitochondria and rough endoplasmic reticulum appear normal ($\times 40,000$); (b) the extracellular quartz particles are not associated with adjacent cytoplasmic changes ($\times 32,000$); (c) much of the quartz is found in membrane bound vesicles sometimes associated with lysosomes (arrow) ($\times 32,000$).

Crocidolite-Treated Cultures

The changes in these cultures were essentially similar to those of the chrysotile-treated cultures, with the exception that no crocidolite fibers were found penetrating or within the nucleus or within deep nuclear invaginations.

Cytotoxicities of Dusts for P388D1 Cells

Table 1 shows the % LDH released from P388D1 cells treated with the mineral dusts. The enzyme release from the nonfibrogenic dust magnetite was similar to that obtained in the nondusted cultures, but the fibrogenic quartz dust was very cytotoxic

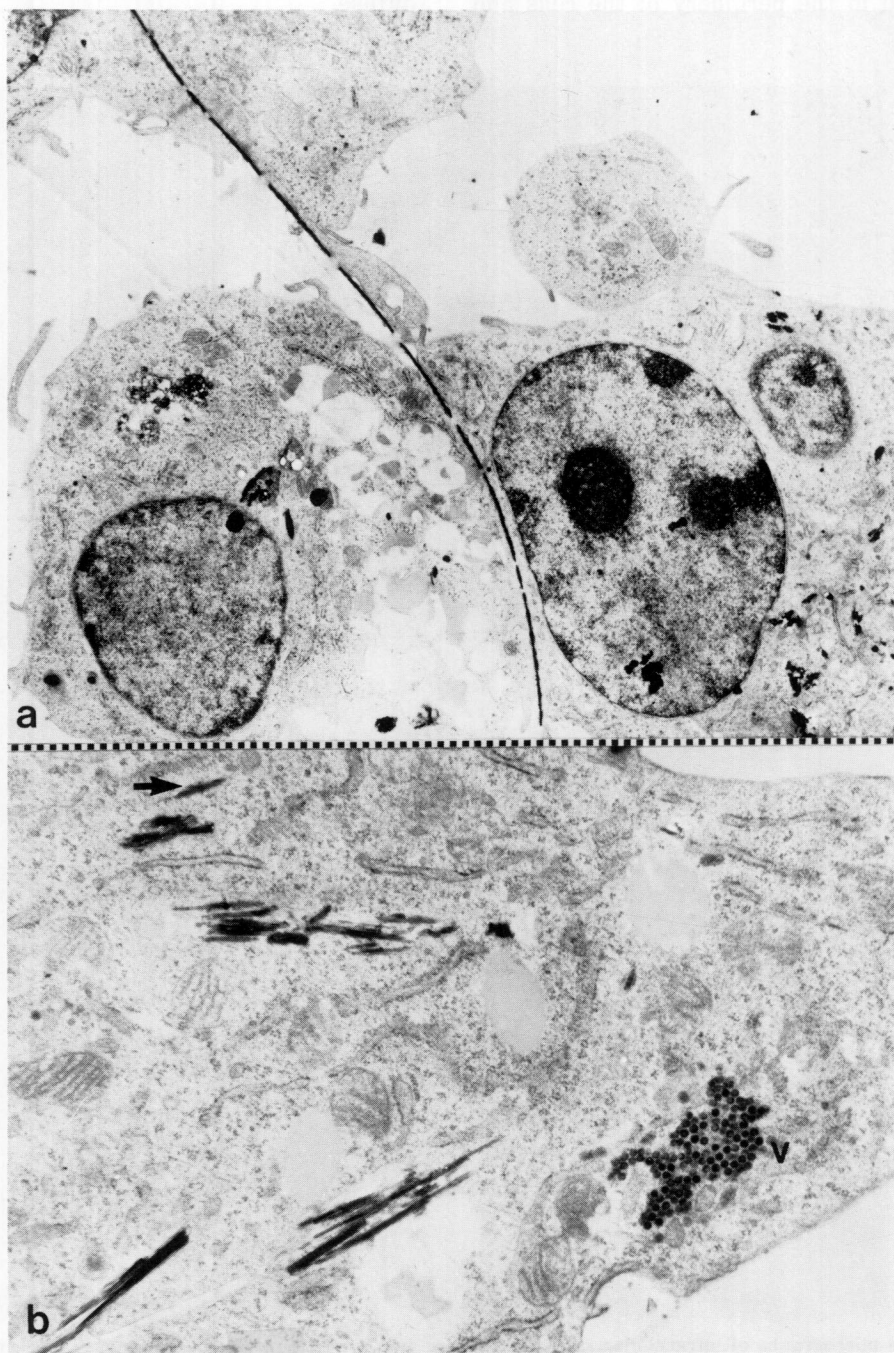


FIGURE 4. Electron micrographs of chrysotile-treated cultures: (a) the cytoplasm appears normal in spite of the presence of mineral fibers ($\times 3,000$); (b) two adjacent cells have partially ingested the same fiber ($\times 14,000$); (c) the cytoplasm appears normal in spite of fibers free in the cytoplasm (arrow). Virus particles are prominent in cytoplasm (V).

towards the cells, releasing large amounts of LDH. The asbestos minerals were modestly cytotoxic.

Discussion

This morphological study confirms the earlier findings of Koren et al. (13) that the P388D1 cell line is macrophagelike in that it possesses acid phosphatase activity and ingests particulate matter. The cells have been shown to contain cytoplasmic virus

particles, some of which contain an RNA core. These viruses do not appear to exert a cytopathic effect, as evidenced by the low level of LDH release from nondusted cells being of similar magnitude to that seen in control primary peritoneal macrophage cultures (1). Cytopathic viruses have been shown to cause a release of LDH from lysosomes (14). Whether the presence of these virus particles modifies the response of the cells in the presence of

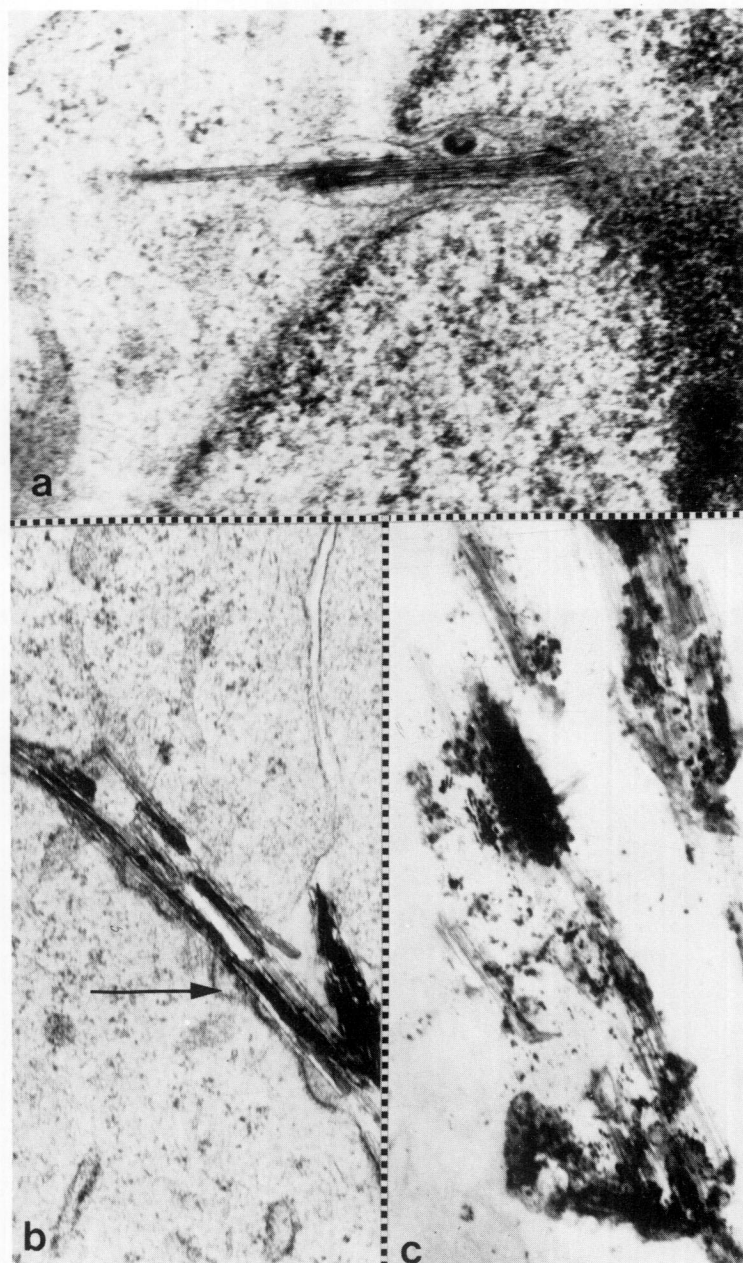


FIGURE 5. Electron micrograph of chrysotile-treated cultures: (a) the chrysotile fiber has penetrated both the phagosome and nuclear envelope ($\times 26,000$); (b) the membrane adjacent to the partially engulfed fibre is associated with coated vesicles (arrow) ($\times 20,000$); (c) phagosomes containing fibers show acid phosphatase activity ($\times 25,000$).

dusts could not be determined. However, the similarities of the response of the P388D1 cell line to that of cultured murine peritoneal macrophages (1) suggest that any effect in terms of cytotoxicity may be slight.

The distribution of the asbestos in the P388D1 cells was similar to that seen in cultured peritoneal

macrophages (3). There seemed little difference in the occurrence of intranuclear fibers in the two macrophage cell systems, in spite of the P388D1 cell line being a dividing cell population. One might have expected intranuclear fibers to be more common in the latter because of the possibility of the fibers becoming incorporated in the nucleus during mitosis

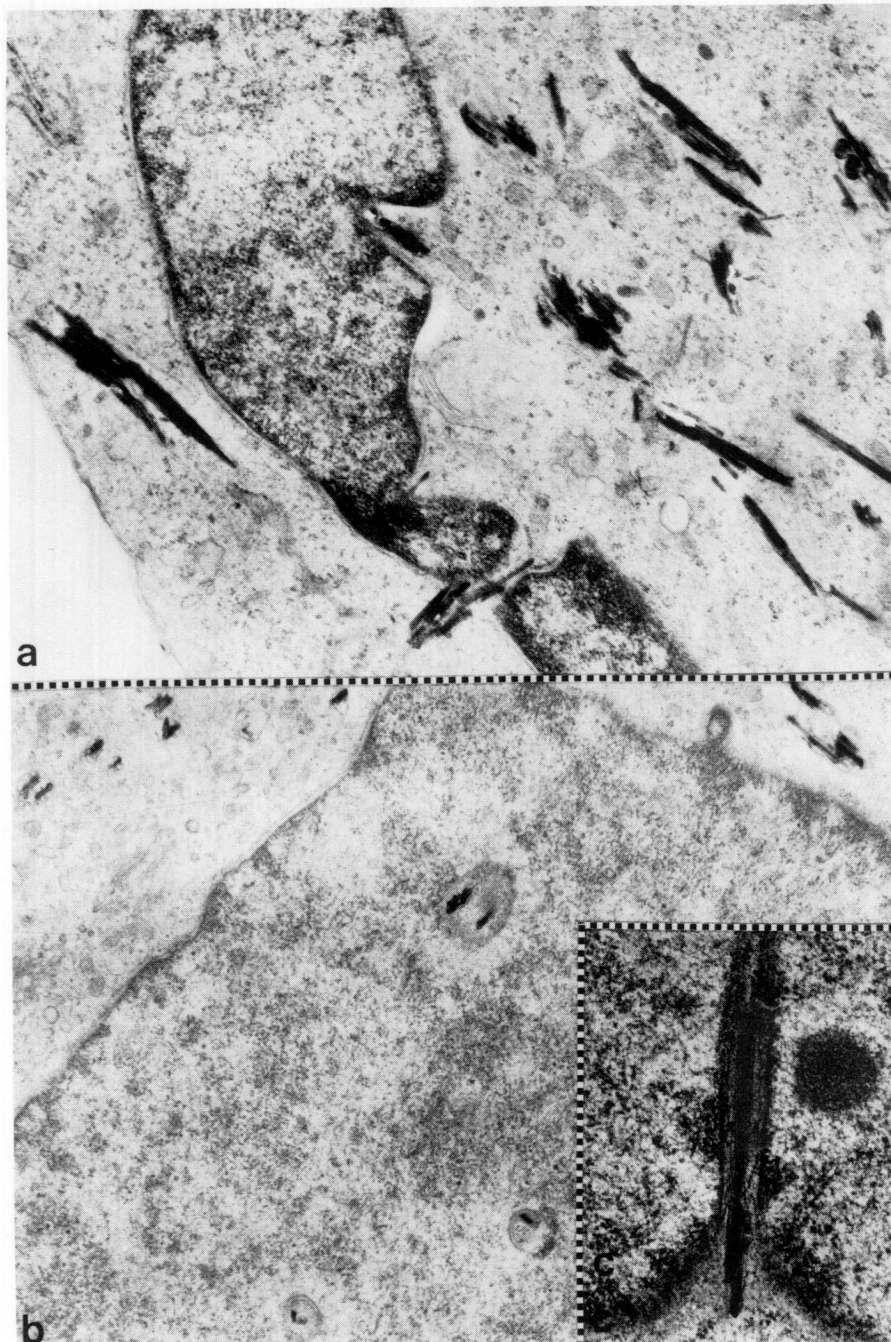


FIGURE 6. Electron micrograph of chrysotile-treated cultures: (a) the nucleus is grossly distorted by adjacent fibers ($\times 18,000$); (b) the nucleus contains cross sections of fibers separated from the chromatin by a portion of nuclear envelope ($\times 31,000$); (c) longitudinal section of fiber contained within a deep invagination of the nucleus ($\times 40,000$).

Table 1. Effect of mineral dusts on LDH enzyme release from P388D1 cells.

Dust treatment	LDH released into culture medium, %
No dust	4.8 \pm 0.2
Magnetite	5.5 \pm 0.8
DQ12 quartz	65.6 \pm 3.0
UICC Canadian chrysotile	29.8 \pm 0.6
UICC crocidolite	18.8 \pm 0.5

when the nuclear envelope is absent. The fibers, however, appear to be pushed to the periphery of the cell. One difference between the P388D1 cells and the peritoneal macrophage cultures was the presence of chrysotile fibers in deep nuclear invaginations in the former, and this may reflect a greater plasticity of the nuclear envelope in these cells. The overall similarity of the effect of asbestos fibers on the two macrophage cell systems may suggest that similar mechanisms operate in both case, i.e., that the fibers exert a mechanical effect in disrupting the cell, by penetrating structures such as the nuclear or phagosomes, by preventing the ordered movement of organelles within the cell, or by disrupting the cytoskeletal framework of the cell.

The inert particulate dust, magnetite, seemed to have no effect on the cells in the context of this experiment. This is in contrast to the silica particles which had a similar distribution with the exception of particles being found free in the cytoplasm. In the silica-treated cultures there was a marked increase in the number of lysed cells. The presence of free silica particles was not associated with changes in the mitochondria and other cell organelles, which is in contrast to the study of Allison (15).

A feature common to both the asbestos and silica-treated cultures was the relatively few aberrant cells, which may suggest that the transition from an ultrastructurally normal cell to an abnormal one is rapid and points to sudden cell death rather than some lingering event.

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